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Identification of androgen receptor phosphorylation in the primate ovary *in vivo*

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Abstract

The androgen receptor (AR) is a member of the nuclear receptor superfamily, and is important for both male and female reproductive health. The receptor is a target for a number of post-translational modifications including phosphorylation, which has been intensively studied *in vitro*. However, little is known about the phosphorylation status of the receptor in target tissues *in vivo*. The common marmoset is a useful model for studying human reproductive functions, and comparison of the AR primary sequence from this primate shows high conservation of serines known to be phosphorylated in the human receptor and corresponding flanking amino acids. We have used a panel of phosphospecific antibodies to study AR phosphorylation in the marmoset ovary throughout the follicular phase and after treatment with GnRH antagonist or testosterone propionate. In normal follicular phase ovaries, total AR (both phosphorylated and non-phosphorylated forms) immunopositive staining was observed in several cell types including granulosa cells of developing follicles, theca cells and endothelial cells lining blood vessels. Receptor phosphorylation at serines 81, 308, and 650 was detected primarily in the granulosa cells of developing follicles, surface epithelium, and vessel endothelial cells. Testosterone treatment led to a modest increase in AR staining in all stages of follicle studied, while GnRH antagonist had no effect. Neither treatment significantly altered the pattern of phosphorylation compared to the control group. These results demonstrate that phosphorylation of the AR occurs, at a subset of serine residues, in a reproductive target tissue *in vivo*, which appears refractory to hormonal manipulations.

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Introduction

The androgen receptor (AR) is a member of the nuclear receptor superfamily and mediates the action of the sex steroids testosterone and dihydrotestosterone (Germann 2002, McEwan 2004). The AR is expressed in reproductive tissues of both males and females, and is known to play important roles in fertility. However, regulation of AR function and the identity of target genes for the receptor in different tissues are less well understood.

Folliculogenesis involves the formation of a multi-cell layer of granulosa cells surrounding the oocyte, which in turn are surrounded by a theca cell layer. The theca cells are responsible for the biosynthesis of androgens, which can be converted to estrogens by the aromatase enzyme that is expressed in granulosa cells in response to FSH stimulation (Fauser & Van Heusden 1997, Hillier 2001, Jamnongjit & Hammes 2006). Androgenic steroids play important roles in follicle growth and survival as

precursors for estrogen biosynthesis and directly through binding to and activation of the AR. The AR has been shown to induce expression of the genes for insulin-like growth factor 1 (IGF1), IGF1 receptor, and FSH receptor, and to augment growth differentiation factor 9 and FSH signaling in granulosa cells (Weil *et al.* 1999, Hickey *et al.* 2005, Walters *et al.* 2007, Lenie & Smitz 2009). Expression of both mRNA and protein for the AR in granulosa cells of the developing follicles has been described in the ovaries from both human (Horie *et al.* 1992, Chadha *et al.* 1994, Rice *et al.* 2007) and a number of non-human primates including rhesus monkey (Weil *et al.* 1998, Chaffin *et al.* 1999), baboon (Hild-Petito & Fazleabas 1997), and the common marmoset, *Callithrix jacchus* (Hillier *et al.* 1997, Saunders *et al.* 2000). AR expression has also been observed and studied in ovaries from mouse (Kimura *et al.* 2007), rat (Tetsuka *et al.* 1995, Tetsuka & Hillier 1996), and sheep (Juengel *et al.* 2006). In these diverse

studies, the consensus findings are that AR protein levels change during follicle development, with the highest levels seen in primary to tertiary stage follicles. Significantly, three complete AR knockout (ARKO) mouse models resulted in reduced fertility in female animals due to defects in folliculogenesis (Yeh *et al.* 2002, Hu *et al.* 2004, Shiina *et al.* 2006, Walters *et al.* 2007).

The AR is subject to a range of post-translational modifications, including phosphorylation on serine (van Laar *et al.* 1991, Gioeli *et al.* 2002, Lin *et al.* 2002, Ponguta *et al.* 2008) and tyrosine (Mahajan *et al.* 2007) residues, and acetylation (Fu *et al.* 2002, Gaughan *et al.* 2002), and sumoylation (Poukka *et al.* 2000, Callewaert *et al.* 2004) on lysine residues. Up to 11 phosphorylated serine and 1 tyrosine residues have been identified to date in cell culture systems after different hormonal or growth factor stimulations (van Laar *et al.* 1991, Gioeli *et al.* 2002, Lin *et al.* 2002, Mahajan *et al.* 2007, Ponguta *et al.* 2008). Phosphorylation of the receptor protein represents a potentially powerful means of regulating receptor levels and function, without the requirement for *de novo* synthesis. However, little is known about the phosphorylation status of the AR in normal target tissues *in vivo*. Recently, a number of phosphoserine-specific antibodies have been described for the AR (Black *et al.* 2004, Taneja *et al.* 2005, Yang *et al.* 2005, Ponguta *et al.* 2008), and the availability of these tools provides the possibility of screening archival tissue samples for cell-specific or developmental changes in receptor phosphorylation, which will be important for a fuller understanding of the role of this chemical modification.

Of the commonly used non-human primate models in reproduction research, the marmoset, *C. jacchus*, has particular advantages with respect to the study of ovarian folliculogenesis. First, marmosets normally ovulate three follicles per cycle (range 1–4) that reach up to 4 mm in diameter so that during the mid-late follicular phase, the ovaries contain a wealth of follicles at different stages of development (Gilchrist *et al.* 2001). Also, the presence of follicular tissue is emphasized because prostaglandin-induced structural luteolysis leads to removal of luteal tissue. Marmoset ovaries, weighing around 150 mg, are ideal to study, being easily accommodated on a microscope slide, so that in ovaries of the mid- and late follicular phase, numerous follicles at different stages of development can be compared within each section. Furthermore, in our laboratory, a number of studies have been performed in which follicular development has been influenced by inhibition of GNRH (Taylor *et al.* 2004, 2007), allowing the secondary effects of this treatment to be investigated (Thomas *et al.* 2007). In the present study, we have addressed the question of changes in and regulation of AR phosphorylation at different stages of development in the marmoset ovary at the normal follicular phase and after manipulation of testosterone levels by suppressing ovarian sex

steroid secretion by GNRH antagonist treatment or by increasing exposure to testosterone by administration of testosterone propionate *in vivo* as described in the rhesus macaque (Vendola *et al.* 1999). By employing immunohistochemical localization using a panel of three phosphoserine-specific antibodies, as well as antibodies recognizing total levels of receptor, we have investigated for the first time the phosphorylation of the AR in the primate ovary.

Results

Eleven phosphorylated serine residues have been identified which are distributed throughout the length of the AR (Fig. 1A; van Laar *et al.* 1991, Gioeli *et al.* 2002, Lin *et al.* 2002, Ponguta *et al.* 2008). In order to determine whether receptor phosphorylation occurs *in vivo* in a target tissue, a panel of antibodies recognizing phosphorylated serines 81, 308, and 650 were used on marmoset ovarian tissue sections. The predicted marmoset AR is 887 amino acids in length and has a theoretical molecular mass of 95 659 kDa. Analysis of the primary amino acid sequence for the marmoset AR revealed strong conservation of serines corresponding to residues in the human AR at positions 81, 94, 308, and 650, which are known to be phosphorylated in the human receptor (Jenster *et al.* 1994, Zhou *et al.* 1995, Blok *et al.* 1998, Lin *et al.* 2001, Zhu *et al.* 2001, Gioeli *et al.* 2002, Rigas *et al.* 2003, Black *et al.* 2004, Wong *et al.* 2004, Chen *et al.* 2006, Kesler *et al.* 2007, Yang *et al.* 2007, Zong *et al.* 2007, Ponguta *et al.* 2008; Fig. 1B). In addition, there was high conservation of flanking amino acids especially a proline at positions 82, 95, 309, and 651 immediately C-terminal of the phosphorylated serine (Fig. 1B). In contrast, the mouse and rat ARs have changes in flanking amino acids, which may impair phosphorylation or detection with phospho-specific antibodies raised against the human sequence. Indeed, none of the phospho-specific antibodies tested appeared to cross-react with the mouse or rat AR in immunohistochemistry (data not shown). Figure 1C shows a western blot of marmoset ovarian tissue extract probed with an antibody against the N-terminus (N20) of the receptor that recognizes both phosphorylated and non-phosphorylated forms of the receptor. The marmoset AR migrated a little slower than the 98 kDa marker. After immunoprecipitation (IP) of the AR, with an antibody against the C-terminal domain (C19), but not a control IgG, forms of the receptor phosphorylated on serines 81, 308, and 650 were detected (Fig. 1D), indicating post-translational modification of the receptor in the primate ovary.

Morphological description of control and GNRH antagonist-treated ovaries has been reported previously (Taylor *et al.* 2004). Briefly, all classes of developing follicles up to the stage of dominant follicles were

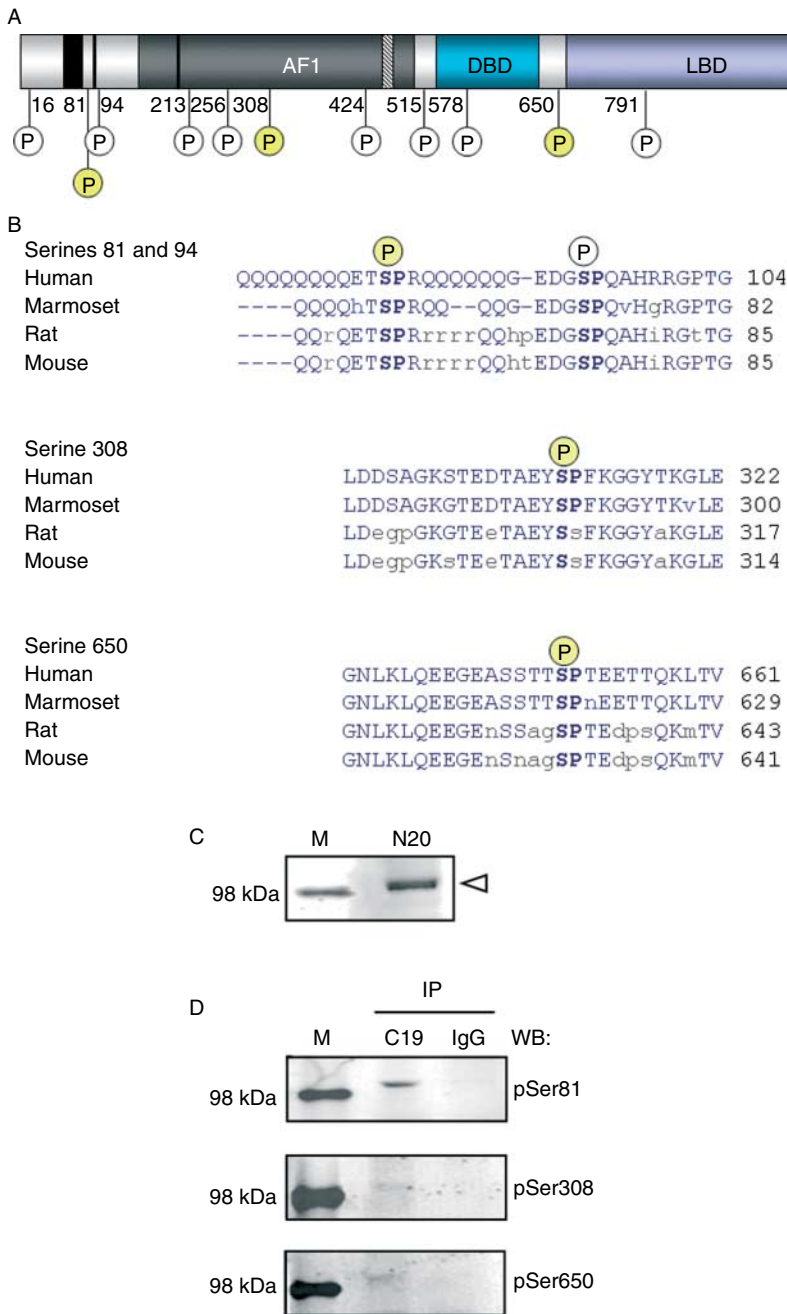


Figure 1 Comparison of primary amino acid sequence of the human, marmoset, and rodent AR. (A) Schematic representation of the human androgen receptor (AR) showing the domain organization and the position of 11 phosphorylated serine residues. The residues analyzed in the present study are highlighted in yellow. LBD, ligand-binding domain; DBD, DNA-binding domain; AF1, activation function 1.

(B) Comparison of the primary amino acid sequence of the human, marmoset, rat, and mouse AR. Only partial amino acid sequence is shown for four phosphorylated serine residues (P). (C) Western blot analysis of total AR (N20 antibody detecting both non-phosphorylated + phosphorylated forms) in a protein extract of the marmoset ovary. M, molecular weight marker. (D) The AR was immunoprecipitated using antibody C19 (against the AR-LBD) and the membranes probed with the phospho-specific antibodies (against phosphoserines 81, 308, and 650). IgG represents a control immunoprecipitation using a non-receptor-specific antibody as a control. IP, immunoprecipitation; WB, western blot.

present in all three groups, while the latter class was present in the day 10 controls only (2–4 per animal). As anticipated, treatment with testosterone propionate was also associated with the presence of large numbers of antral follicles and the absence or reduction in numbers of dominant follicles. In order to determine the location of the phosphorylated AR in the ovary, immunohistochemistry was undertaken on whole tissue sections. Examination of ovaries at the two control stages of the cycle revealed a similar intensity of staining within each class of follicle, irrespective of stage. Because healthy tertiary follicles were most abundant in the day 5

ovaries, these were selected for semi-quantitative analysis and only pre-ovulatory follicles from the day 10 controls were scored. As reported previously (Hillier *et al.* 1997, Saunders *et al.* 2000), in the ovaries of control marmosets, AR-positive nuclear staining was observed in the granulosa cells at all stages of follicle development when sections were incubated with an antibody directed against the N-terminus of the mature protein (Fig. 2A N20). AR-positive staining in granulosa cells increased as follicles developed (Fig. 2A panels a and d and B), and began to decrease during early and late atresia (data not shown). The theca cell layer, stroma,

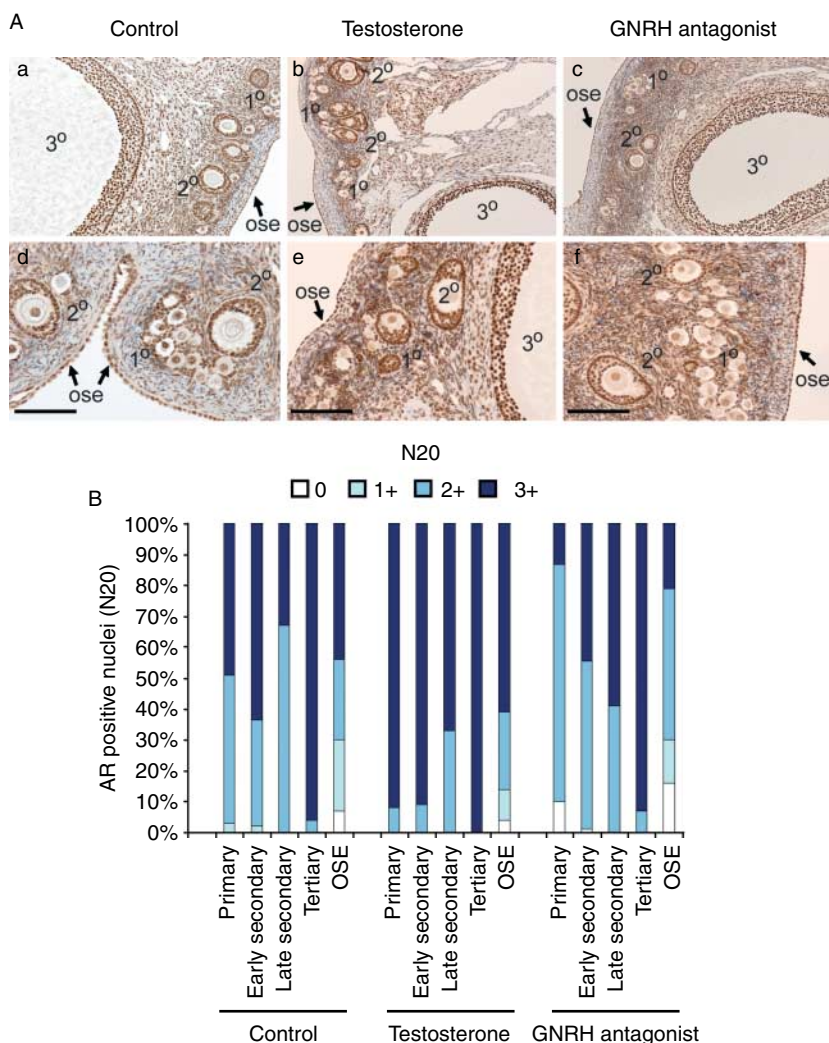


Figure 2 AR expression in the marmoset ovary. (A) Immunohistochemistry staining for total AR using an antibody against the first 20 amino acids (N20). Panels a and d, control group; panels b and e, after treatment with testosterone propionate; and panels c and f, after treatment with GnRH antagonist. Panels a–c and d–f represent $\times 10$ and $\times 20$ magnification respectively; size bar = 100 μm . 1°, 2°, and 3° examples of primary, secondary, and tertiary follicles are shown; OSE, ovarian surface epithelium. (B) Scoring for the relative strength of staining for total AR in control animals (day 5); after treatment with GnRH antagonist on day 0; and after treatment with testosterone propionate on day 0. Ovaries from four animals were analyzed for each condition, and scoring was done as detailed in Materials and Methods. Briefly, only primary/transitional and secondary follicles with a clearly visible oocyte were scored as follows: 0, no detectable staining; 1+, 2+, and 3+, weak, moderate, and strong staining respectively. The percentage of granulosa cells at each intensity for primary, secondary, and tertiary follicles has been plotted together with the percentage of OSE.

and regions of the ovarian surface epithelium (OSE) also all stained positive for AR (Fig. 2A panels a and d).

Treatment with testosterone propionate resulted in strong staining for total AR in primary follicles through to tertiary follicles and the OSE (Fig. 2A panels b and e and B). From the semi-quantitative scoring of the AR staining, it could be seen that there was an overall increase in the levels of receptor at all stages of follicle growth, especially primary and secondary follicles (Fig. 2B).

To investigate the effect of GnRH antagonist treatment on AR phosphorylation, ovarian sections from animals treated on day 0 were stained for total AR or selected phosphoserine modifications. After GnRH antagonist treatment, AR staining was observed in all stages of follicle present, and again predominantly in granulosa and theca cells of primary, secondary, and tertiary follicles and reduced in atretic follicles (Fig. 2A panels c and f and B). This treatment results in failure of emergence of dominant follicles (Taylor *et al.* 2004).

Phosphorylated forms of the AR were similarly observed at all stages of follicle growth. The pattern of

phosphorylated serine 81 essentially mirrors that of total AR with most intense immunopositive staining seen in the secondary and tertiary follicles and the OSE (Fig. 3A panel a and B). Nuclear staining was also detected in the theca cell layer.

Phosphorylation on serine 308, also present in the AR-amino terminal domain (NTD), was restricted mainly to the granulosa cells of secondary and tertiary follicles with little or no staining of primary (Fig. 4A panel a and B) or pre-ovulatory follicles (data not shown). Phosphorylation of serine 308 was also more punctate, with some strongly staining granulosa cells (Fig. 4).

The pattern of phosphorylation of serine 650, located within the hinge domain, was observed in all stages of follicle development from primary onwards, with the most intense staining seen in secondary and tertiary follicles (Fig. 5A panel a and B). Immunopositive staining was reduced, but still observable in pre-ovulatory/dominant follicles and reduced or absent in atretic follicles (data not shown).

Staining for total AR and phosphoserines 81 and 650 was also observed in regions of the OSE, while staining

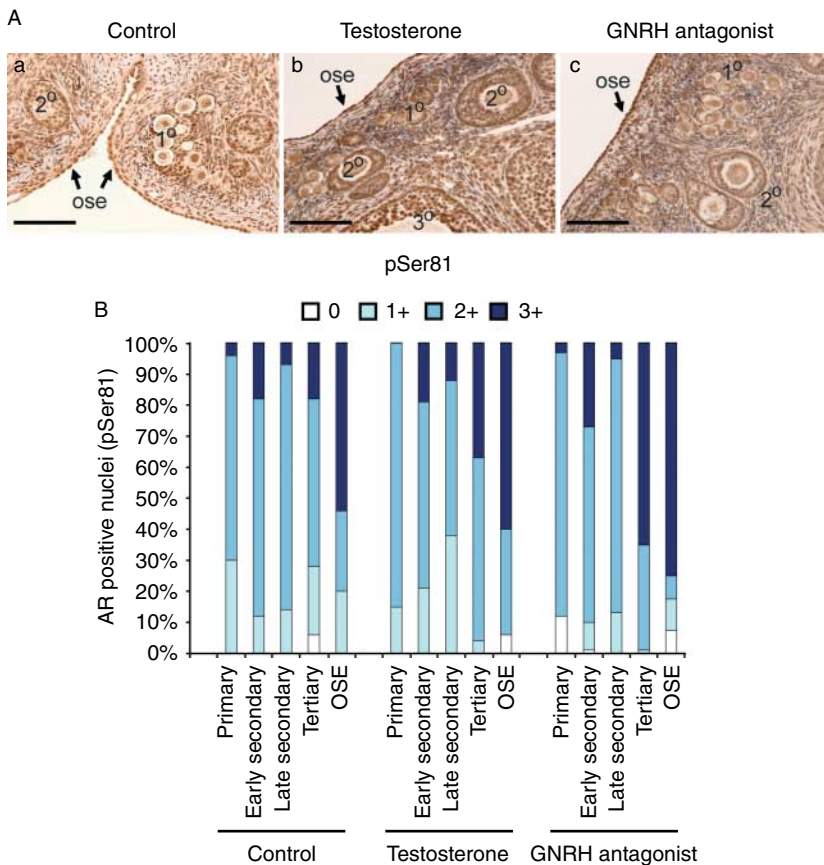


Figure 3 Detection of AR phosphorylated on serine 81. (A) Immunohistochemistry staining for AR using an antibody against phosphoserine 81 (pSer81). Panel a, control animal (day 5); panel b, after treatment with testosterone propionate; and panel c, after treatment with GNRH antagonist. Panels a–c represent $\times 20$ magnification; size bar = 100 μm . 1°, 2°, and 3° examples of primary, secondary, and tertiary follicles are shown; OSE, ovarian surface epithelium. (B) Scoring for the relative strength of staining for total AR in control animals (day 5); after treatment with GNRH antagonist on day 0; and after treatment with testosterone propionate on day 0. Ovaries from four animals were analyzed for each condition, and scoring was done as detailed in Materials and Methods. Briefly, only primary/transitional and secondary follicles with a clearly visible oocyte were scored as follows: 0, no detectable staining; 1+, 2+, and 3+, weak, moderate, and strong staining respectively. The percentage of granulosa cells at each intensity for primary, secondary, and tertiary follicles has been plotted together with the percentage of OSE.

for phosphoserine 308 was more patchy with only some cells staining positive (Figs 2–5). It is also noteworthy that positive staining for total and phosphorylated forms of the AR was also observed in about 30% of endothelial cells lining blood vessel walls (Fig. 6). Taken together, the data indicate that the AR phosphorylated on specific serine residues is detectable in cells of the primate ovary.

As a control for staining specificity of the anti-phosphoserines 81, 308, and 650 antibodies, positive staining was efficiently competed with the respective phosphoserine peptide (Fig. 7 panels a–f). AR-positive immunostaining using the N20 antibody was not significantly impaired in the presence of these peptides (Fig. 7 panel h and data not shown).

The phosphorylation of the AR on serines 81, 308, and 650 was not significantly altered by manipulating the hormonal environment (Figs 3–5); thus, antagonizing GNRH action or raising testosterone levels cause morphological changes in the primate ovary, but had little impact on the phosphorylation status of the AR. Overall AR phosphorylation on serines 81, 308, and 650 was detected in several cell types of the primate ovary, most notably the granulosa cells of developing follicles and in regions of the OSE, together with theca and stromal cells and a proportion of endothelial cells lining blood vessels.

Discussion

The marmoset model has proved useful in understanding primate ovarian biology and has the advantage that extensive studies have been carried out on manipulating the hormone environment *in vivo*. In this study, we have confirmed and extended previous work reporting AR expression in the primate ovary. Nuclear immunopositive staining of total AR was observed strongly in the granulosa cells, but also in the OSE, theca cell layer and stroma cells, and in a proportion of endothelial cells lining blood vessels. These findings are in good agreement with earlier work describing the similar expression patterns of the AR mRNA and/or protein in the rhesus monkey, marmoset, and human ovary (Chadha *et al.* 1994, Hillier *et al.* 1997, Weil *et al.* 1998, Saunders *et al.* 2000). Significantly, we show that phosphorylated forms of the AR are present primarily in granulosa cells, but also in other cell types including the OSE and endothelial cells.

In addition to their presence in the hormone-producing cells of the follicle, the presence of AR and its phosphorylated forms in the OSE and a subset of ovarian endothelial cells indicate that ovarian blood vessels and OSE may be a target for androgen action. AR has been localized in endothelial cells in a number of tissues (Pelletier *et al.* 2000, Gonzales *et al.* 2007), but its

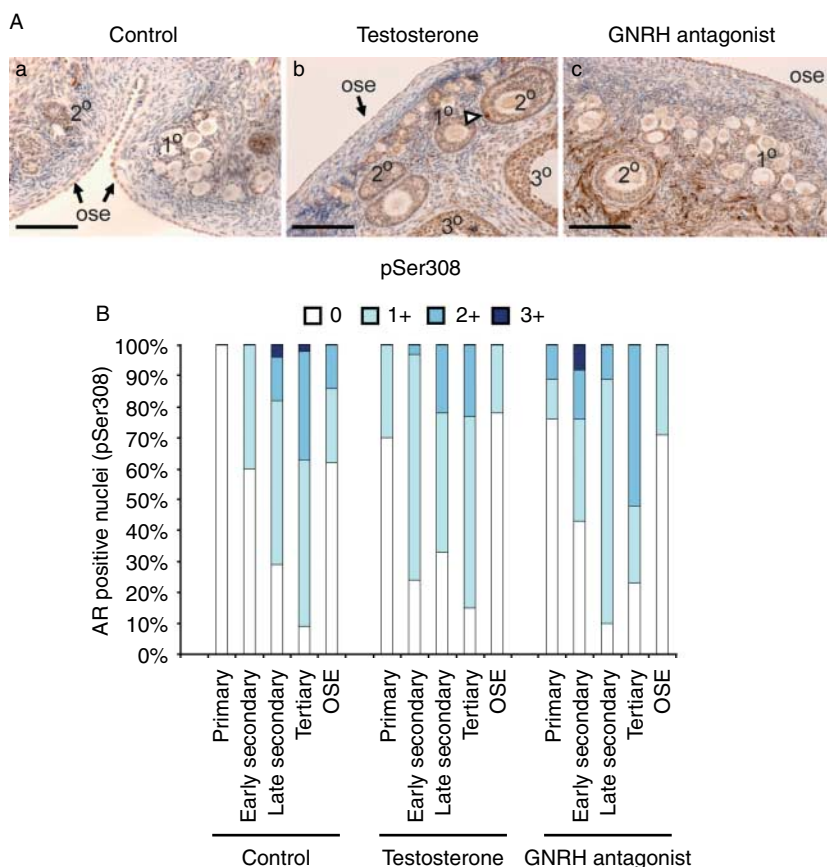


Figure 4 Detection of AR phosphorylated on serine 308. (A) Immunohistochemistry staining for AR using an antibody against phosphoserine 308 (pSer308). Panel a, control animal (day 5); panel b, after treatment with testosterone propionate; and panel c, after treatment with GNRH antagonist. Panels a–c represent $\times 20$ magnification; size bar = 100 μm . 1°, 2°, and 3° examples of primary, secondary, and tertiary follicles are shown; OSE, ovarian surface epithelium. Open arrow head represents an example of a strongly staining cell. (B) Scoring for the relative strength of staining for total AR in control animals (day 5); after treatment with GNRH antagonist on day 0; and after treatment with testosterone propionate on day 0. Ovaries from four animals were analyzed for each condition, and scoring was done as detailed in Materials and methods. Briefly, only primary/transitional and secondary follicles with a clearly visible oocyte were scored as follows: 0, no detectable staining; 1+, 2+, and 3+, weak, moderate, and strong staining respectively. The percentage of granulosa cells at each intensity for primary, secondary, and tertiary follicles has been plotted together with the percentage of OSE.

role in influencing vascular development and function is unclear. Several other factors have been shown to have major actions on these cells (Fraser & Duncan 2009), and it is likely that these potential sites of action are of much less significance than the role of androgen on the granulosa cells. Several studies have observed the expression of the AR in the OSE and the epithelial cells of inclusion cysts (see Saunders *et al.* 2000, Edmondson *et al.* 2002). Also androgen stimulation of primary cultures of human OSE cells increased cell proliferation (Edmondson *et al.* 2002), suggesting that the OSE is an androgen target.

In cell culture systems, phosphorylation of serines 81, 308, and 650 has been shown to be induced by androgen treatment (see Gioeli *et al.* 2002). Furthermore, a number of kinases capable of phosphorylating these residues have been identified *in vitro*. In the case of phosphorylation of serines 81 and 308, the cell cycle-dependent kinases 1 and 11 have been implicated (Chen *et al.* 2006, Zong *et al.* 2007). Phosphorylation of serine 308 has been associated with repression of AR activity by cyclin D3/CDK11 (Zong *et al.* 2007). Furthermore, targeting the receptor to the cytoplasmic or nuclear compartments resulted in a bias of nuclear phosphorylation of serines 81 and 308 (Kesler *et al.* 2007), which the authors suggest plays a role in nucleocytoplasmic shuttling. However, earlier studies

failed to see a role for serine 81 phosphorylation in transactivation assays (Jenster *et al.* 1994, Zhou *et al.* 1995) or nuclear/cytoplasmic distribution (Jenster *et al.* 1994). Consistent with a lack of effect directly on transactivation, Paschal and co-workers (Black *et al.* 2004) reported that serine 81 phosphorylation occurs subsequent to receptor–co-regulatory protein interactions. In the absence of an unambiguous role for phosphorylation of the individual serines at positions 81 and 308, it is tempting to speculate that what matters is the net phosphorylation of the receptor rather than modification of individual residues. Alternatively, it is conceivable that the functional consequences of phosphorylating individual serines maybe cell and/or gene specific. Further work will be necessary to distinguish these possibilities.

Serine 650 can be phosphorylated *in vitro* by protein kinase A and protein kinase C and by members of the MAPK family, including the stress-induced kinases p38 α and JNK (Gioeli *et al.* 2002, 2006). Although phosphorylation of this residue is well documented, the functional consequences of this modification remain a subject of debate. Initial studies reported a modest impairment of transactivation when the serine is mutated to alanine (S650A; Zhou *et al.* 1995), and the functionally similar mutation to glycine (S650G) is associated with mild androgen insensitivity syndrome

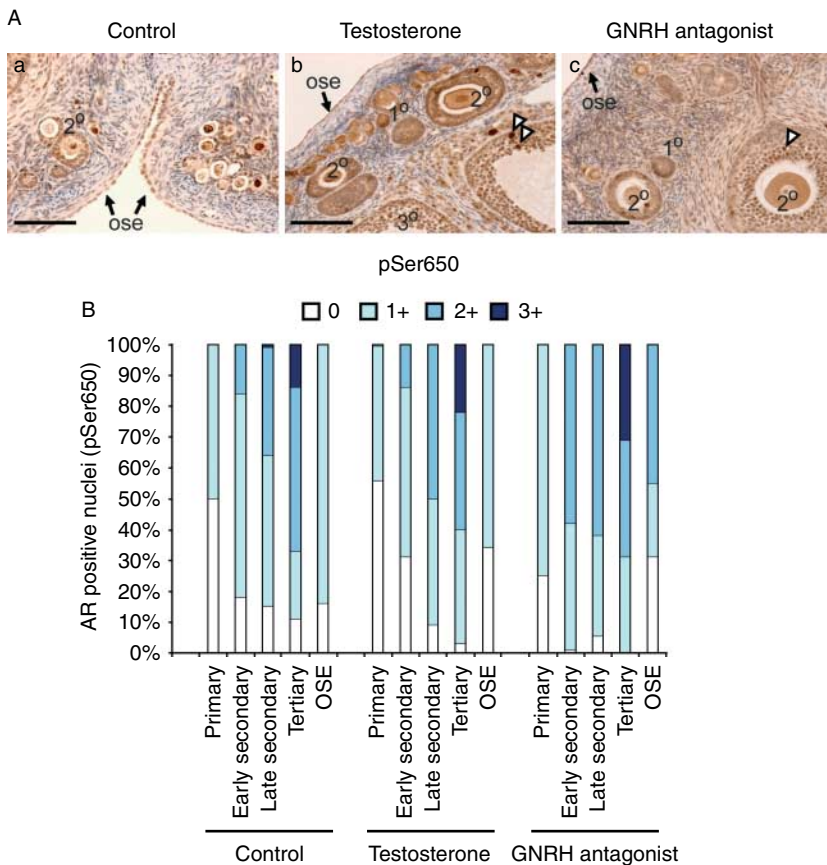


Figure 5 Detection of AR phosphorylated on serine 650. (A) Immunohistochemistry staining for AR using an antibody against phosphoserine 650 (pSer650). Panel a, control animal (day 5); panel b, after treatment with testosterone propionate; and panel c, after treatment with GNRH antagonist. Panels a–c represent $\times 20$ magnification; size bar = 100 μm . 1°, 2°, and 3° examples of primary, secondary, and tertiary follicles are shown; OSE, ovarian surface epithelium. Open arrow head represents an example of a strongly staining cell. (B) Scoring for the relative strength of staining for total AR in control animals (day 5); after treatment with GNRH antagonist on day 0; and after treatment with testosterone propionate on day 0. Ovaries from four animals were analyzed for each condition, and scoring was done as detailed in Materials and methods. Briefly, only primary/transitional and secondary follicles with a clearly visible oocyte were scored as follows: 0, no detectable staining; 1+, 2+, and 3+, weak, moderate, and strong staining respectively. The percentage of granulosa cell at each intensity for primary, secondary, and tertiary follicles has been plotted together with the percentage of OSE.

(Ferlin *et al.* 2006) and male infertility (Zuccarello *et al.* 2008). On the other hand, others have found no major changes in protein–protein interactions or transactivation activity with the same mutation (Wong *et al.* 2004). More recently, Gioeli *et al.* (2006) have demonstrated that serine 650 phosphorylation regulates nuclear export, with the non-phosphorylatable S650A mutant receptor retained in the nucleus and a concomitant increase in a target gene expression. Similarly, Chen *et al.* (2009) reported that serine 650 phosphorylation was a target for protein phosphatase 1, and it was involved in nuclear export and subsequent receptor turnover. However strikingly, in the present study, immunodetection of phosphoserines 81, 308, and 650 *in vivo* appeared predominantly nuclear, although some cytoplasmic staining was also observed.

The gonadotropins FSH and LH play critical roles in folliculogenesis, theca and granulosa cell functions, and ovulation, including the LH-stimulated activation of androgen biosynthesis in theca cells (Fauser & Van Heusden 1997, Hillier 2001, Jamnongjit & Hammes 2006). The release of these peptide hormones from the pituitary is in turn regulated by GNRH. Treatment of animals with a GNRH antagonist on day 0 of the ovulatory cycle results in inhibition of development of large dominant follicles and suppression of ovarian sex hormone secretion (Taylor *et al.* 2004). The likely

deprivation of the follicles of intra-ovarian androgen might have been expected to result in decreased expression of the AR and impact directly on AR phosphorylation. Conversely, treatment with testosterone propionate throughout the follicular phase might have been expected to increase expression and phosphorylation. An increase in AR in the testosterone propionate ovaries was confirmed; however, our semi-quantitative scoring did not reveal substantive changes in any other parameters following these treatments. This indicates that AR phosphorylation in ovarian compartments is not closely regulated by testosterone, at least over the 10-day period of the present study. Interestingly, an increase in receptor mRNA has been observed in the granulosa cells in the rhesus monkey after testosterone treatment and was correlated with cell proliferation (Weil *et al.* 1998). Although phosphorylation on serines 81, 308, and 650 is refractory to hormone manipulation *in vivo*, it does not necessarily follow that phosphorylation plays no role in receptor function in the primate ovary, and this is an area of ongoing investigation.

To date, phosphorylation of the AR has been studied almost exclusively in cell culture-based systems with exogenously expressed receptor or in the bone-derived metastatic prostate cancer cell line LNCaP, containing a mutant AR. In the present study, we have uniquely

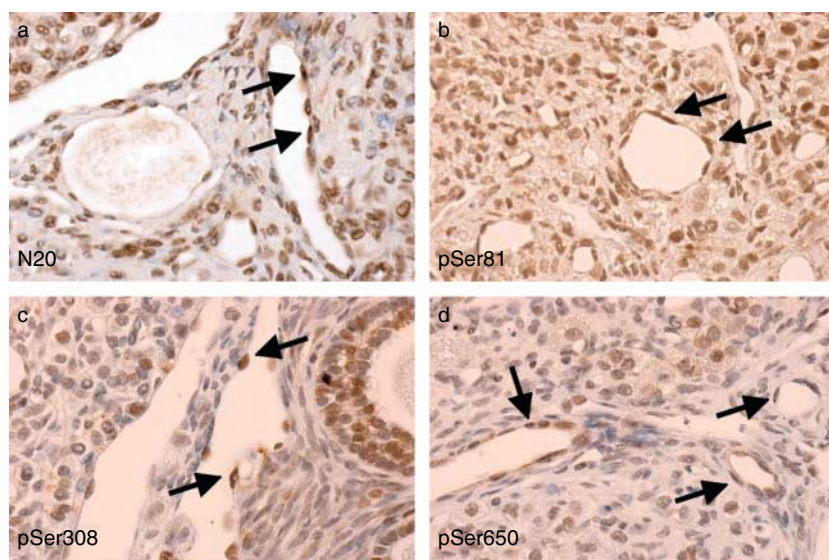


Figure 6 Total AR and phosphorylated forms in endothelial cells. Detection of total AR (N20) and phosphorylated forms (serines 81, 308, and 650) in a proportion of endothelial cells (→) lining blood vessels. Staining is of an ovary from a control animal.

demonstrated *in vivo* phosphorylation of the AR in a reproductive tissue. These findings complement and expand upon work describing the presence of phosphorylated serine 213 in the developing and adult prostate gland (Taneja *et al.* 2005) and in clinical samples of prostate cancer (McCall *et al.* 2008). Taken together with the present study, these data indicate that phosphorylation of the AR has the potential to regulate receptor function in male and female reproductive tissues and in disease.

Materials and Methods

Antibodies

Total AR protein expression was detected using a rabbit polyclonal antibody (N20, sc-816) raised against the receptor N-terminus (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Antibodies recognizing phosphoserines 81 (Millipore Ltd (UK), Watford, UK), 308 (sc26406-R; Santa Cruz Biotechnology Inc.), and 650 (ab47563; Abcam, Cambridge, UK) were all rabbit polyclonals raised against phosphoserine-containing peptides derived from the human AR sequence.

Animals, treatments and tissue collection

As the objective was to determine changes in receptor localization during follicular development, control ovaries were examined at two stages of the follicular phase of the ovulatory cycle. To synchronize the timing of events during the cycle, adult female marmosets were injected with 1 µg prostaglandin PGF_{2α} analog (cloprostenol, Planate; Coopers Animal Health Ltd, Crewe, UK), i.m. on day 13–15 of the luteal phase to induce luteolysis. The day of prostaglandin injection was designated as follicular day 0. This method of synchronizing follicular recruitment was followed by follicle selection on day 5 and ovulation between day 9 and 11. Thus, ovaries were studied on day 5 as they contained an abundance of healthy

tertiary follicles and on day 10 of the cycle when pre-ovulatory follicles were present. To study the effects of sex hormone ablation on the one hand and hyperstimulation with testosterone on the other hand, marmosets ($n=4$ per group) were injected with PGF_{2α} analog as the controls, but were treated on day 0 with either a GNRH antagonist, Teverelix [N-Ac-D-Nal¹, D-pCl-Phe², D-Pal³, D-(Hci)⁶, Lys(iPr)⁸, D-Ala¹⁰] GNRH (Europeptides, Argenteuil, France), 12 mg/kg, s.c., to block gonadotropin secretion (Taylor *et al.* 2004), or testosterone propionate (AMS Biotechnology, Abingdon, UK), 20 mg, s.c., in 200 µl vegetable oil repeated on days, 1, 3, 5, 7, and 9. On day 10, animals were killed as described previously (Taylor *et al.* 2004), and ovaries were collected into 4% neutral-buffered formalin for 24 h before being transferred into 70% ethanol, dehydrated, and embedded in paraffin according to standard procedures. Ovaries from control and GNRH antagonist treatments had been stored at room temperature in paraffin blocks having been generated from previously described experiments (Taylor *et al.* 2004, 2007). Treatment with testosterone propionate was carried out in accordance with the Animals (Scientific Procedures) Act, 1986, and approved by the Local Ethical Review Process Committee. Ovarian sections (5 µm) were cut onto SuperFrost slides (BDH, Merck Co., Inc.), and stained with hematoxylin and eosin or subjected to immunohistochemistry as described below.

Marmoset androgen receptor

The coding sequence of the marmoset AR gene and conceptual translation were determined in two stages. Initially, sequences of human AR exons were used to blast the *C. jacchus* genome-sequencing database at the Genome Sequencing Center at Washington University Medical School, St Louis, MO, USA (<http://genome.wustl.edu>). High scoring segments were employed to download sequencing traces from the National Center for Biotechnology Information Trace Archive, and these were aligned using ClustalW2 (Larkin *et al.* 2007). Sequences shared between three and six traces were assembled to create contiguous regions of the gene, which were used to confirm

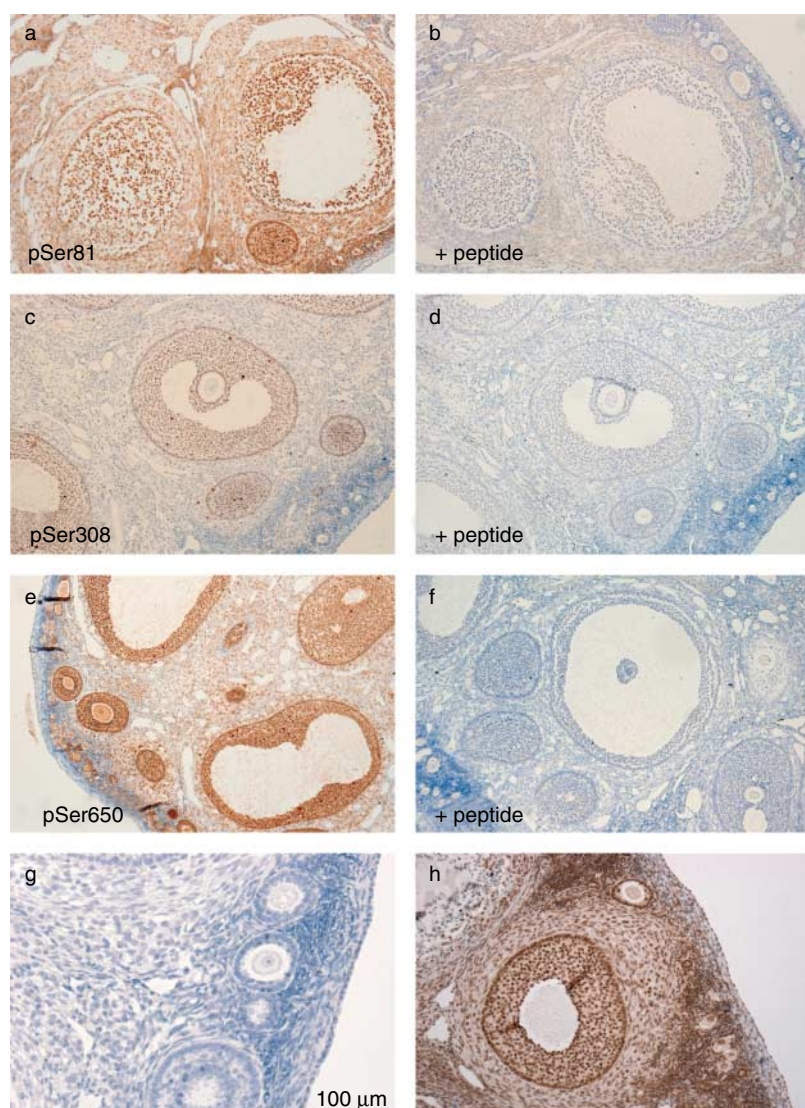


Figure 7 Specificity of phospho-specific antibodies. Panels a, c, and e show staining of follicles for phosphoserines 81, 308, and 650 respectively. Panels b, d, and f represent staining in the presence of competing amounts of the cognate phosphopeptide. Panel g represents no primary antibody. Panel h represents staining for AR with N20 in the presence of an excess of phosphoserine 81 peptide. Magnification $\times 10$.

intron/exon splice junctions. The coding sequence was subsequently translated to give a predicted protein primary sequence. Most recently, the coding sequence was confirmed by comparison to the 6 \times whole-genome shotgun supercontig 3.2.

Immunohistochemistry

After dewaxing and rehydration of tissue sections, antigen retrieval was achieved in 50 mM glycine–EDTA buffer (pH 8) for 5 min in a pressure cooker. Slides were then blocked with 3% (v/v) H_2O_2 in methanol for 30 min, and washed with water and then with 50 mM Tris–HCl (pH 7.4) and 150 mM NaCl (TBS). Slides were blocked with 20% normal goat or horse serum and 5% BSA in TBS for up to 1 h prior to incubation overnight at 4 °C with primary antibodies. Antibodies were used at the following dilutions: N20 (1:200, 3 nM); pSerine 81 (1:50, 0.07 μM); pSerine 308 (1:100, 0.01 μM); and pSerine 650 (1:25, 0.27 μM). Slides were then washed twice with TBS, and incubated with goat or horse anti-rabbit secondary antibody with polymerized reporter enzyme staining system (EnVision; Dako

Corporation, Copenhagen, Denmark; or ImmPress, Vector Labs, Peterborough, UK) for up to 1 h at room temperature. After two wash steps with TBS, DAB substrate was added, and the reaction developed for 2–4 min. Images were captured using a Provis microscope (Olympus Corp., London, UK). All ovaries were subjected to immunohistochemistry in the same run.

Competing peptides containing phosphorylated serine residues were custom synthesized by EZBiolab (Westfield, IN, USA): pSerine 81 QQQQQQETpSPRQQ; pSerine 308 KSTEDTAETpSPFKG; and pSerine 650 EEGEASSTpSPTEE. Peptides were added to antibody solutions at concentrations ranging from 0.03 to 2.58 mM prior to tissue incubation.

Analysis of sections

Stages of follicular development were defined as described previously (Taylor *et al.* 2004, 2007), i.e. primordial (oocyte surrounded by a few flattened granulosa cells), transitory (oocyte surrounded by flattened granulosa cells and at least one cuboidal granulosa cell), primary (oocyte surrounded by

a complete layer of cuboidal cells), early secondary (two to four granulosa cell layers, no antrum), late secondary (more than four granulosa cell layers, no antrum), tertiary (follicles containing an antrum), and dominant (large antral follicles, > 2 mm). Follicles were classified as healthy if they contained a normal-shaped oocyte surrounded by granulosa cells that were regularly apposed on an intact basement membrane with normal appearance of granulosa cell nuclei without signs of pycnosis. For examination of primordial to late secondary follicles, only those with a visible oocyte containing a nucleus were considered to ensure proper follicular classification. Follicles were classified as atretic when more than 5% of granulosa cells showed signs of pycnosis and detachment of cells from the basement membrane. The numbers of follicles analyzed were as follows: controls (transitory/primary, $n=262$; early secondary, $n=154$; late secondary, $n=29$; tertiary, $n=200$; dominant, $n=26$; and atretic, $n=19$); GnRH antagonist-treated (transitory/primary, $n=287$; early secondary, $n=148$; late secondary, $n=48$; tertiary, $n=142$; dominant, $n=0$; and atretic, $n=21$), and testosterone propionate-treated (transitory/primary, $n=274$; early secondary, $n=137$; late secondary, $n=36$; tertiary, $n=103$; dominant, $n=2$; and atretic, $n=39$).

Sections were analyzed by two observers blinded to the treatment category. The degree of staining for AR protein was based upon a modified HSCORE. Thus, intensity of staining in granulosa cells and OSE was classed as 1, weak; 2, moderate; 3, strong. The percentage of granulosa cells and OSE at each intensity was then calculated. Theca and endothelial cells were also examined.

Immunoprecipitation and western blot analysis of the marmoset AR

For IP, a single normal ovary was homogenized using RIPA buffer (1% Triton X-100, 0.25% sodium deoxycholate, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA) containing protease inhibitors (Complete Mini protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (2 mM activated Na_3VO_4 ; 50 mM NaF; 20 mM β -glycerophosphate disodium; phosphatase inhibitor cocktail containing cantharidin, bromotetramisole, and microcystin-LR (Sigma-Aldrich)). Protease and phosphatase inhibitors were added to the lysis buffer just before homogenization. The AR was immunoprecipitated from an aliquot of 0.5 mg protein/ml using 2.5 μg of anti-human AR (C-19, Santa Cruz Biotechnology Inc.) and protein A affinity resin (Sigma-Aldrich). For each IP experiment, appropriate negative controls (rabbit IgG and rabbit serum) were conducted simultaneously. Prior to the incubation with the C-19 antibody, the tissue homogenate was precleared with protein A resin. The final pellets were washed three times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ PBS. Purified immune complexes associated with AR were mixed with 2 \times electrophoresis sample buffer, boiled, and then resolved by SDS-PAGE. These complexes were further analyzed by western blotting using antibodies raised against AR and specific serine phosphorylation sites. Briefly, membranes were blocked with 5% non-fat dry milk/TBS for 1 h followed by incubation with primary antibodies

diluted in 5% milk/TBS for 2 h at room temperature: N20 (1:100), pSerine81 (1:500), pSerine308 (1:200), and pSerine650 (1:200). The secondary antibodies such as IRDye680-conjugated affinity-purified goat anti-mouse IgG or IRDye800CW-conjugated affinity-purified goat anti-rabbit IgG (Li-COR Biosciences, Cambridge, UK) at 1:1000 dilution were incubated for an hour. Fluorescence was analyzed using the Odyssey Infrared Imaging system from LI-COR Biosciences. To verify the specificity of AR, phosphoserine antibodies blocking peptides and λ protein phosphatase (100 U at 37 °C) treatment were used.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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